Cryptolepis sanguinolenta: an Ethnobotanical Approach to Drug Discovery and the Isolation of a Potentially Useful New Antihyperglycaemic Agent

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Evidence has been published that a wide array of plant-derived active principles, representing numerous classes of chemical compounds, demonstrate activity consistent with their possible use in the treatment of patients with Type 2 diabetes mellitus (DM). Despite these interesting observations, to date, metformin is the only ethical drug approved for treatment of Type 2 DM derived from a medicinal plant. Why is this so, given the fact that higher plants are such a potential source of new drugs? The answer to this rhetorical question may lie in the reliance of most pharmaceutical companies on random, *in vitro*, mechanism-based, high throughput screening in the initial phases of plant drug research. In this article we describe an alternative pathway to discovery of drugs for the treatment of Type 2 DM: on based on an ethnomedical approach, involving ethnobotany and traditional medicine. In particular, we present evidence that cryptolepine, an indoloquinolone alkaloid isolated from *Cryptolepis sanguinolenta*, significantly lowers glucose when given orally to a mouse model of diabetes. The antihyperglycaemic effect of cryptolepine leads to a significant decline in plasma insulin concentration, associated with evidence of an enhancement in insulin-mediated glucose disposal. Finally, cryptolepine increases glucose uptake by 3T3-L1 cells. These data permit us to conclude that an ethnobotanical approach to drug discovery can identify a potentially usful drug for the treatment of Type 2 DM. © **1998 John Wiley & Sons, Ltd.**

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Introduction

Pharmaceutical research conducted over decades has shown that natural products are a potential source of novel molecules for drug development.[1,2] In this context, a wide array of plant-derived active principles, representing numerous classes of chemical compounds, demonstrate activity consistent with their possible use in the treatment of Type 2 (non-insulin-dependent) diabetes mellitus (DM).[3–6] However, metformin is the only ethical drug so far approved for treatment of Type 2 DM derived from a medicinal plant (*Galegos officinalis*) historically used to treat diabetes. One possible explanation for this paradox is the reliance of most pharmaceutical companies on random, *in vitro*, mechanism-based, high throughput screening in the initial phases of plant drug discovery. This is not the only approach to discovery of drugs for the treatment of Type 2 DM, and we have recently described an alternative pathway based on an ethnomedical approach, involving ethnobotany and traditional medicine, utilizing modern techniques of natural product chemistry and rodent models of Type 2 DM to isolate and identify compounds with antihyperglycaemic activity.[7] Briefly, the ethnomedical approach begins with an interaction between a Western-trained physician and botanist with an indigenous healer. Based upon the use of clinical vignettes, information is acquired on the plants the healer has found useful for treatment of patients with the signs and symptoms of Type 2 DM. This information is evaluated, along with any available use of a given plant so identified, and a decision made as to whether or not it seems useful to proceed further. If a positive decision is reached, a substantial supply of the plant is gathered, several extracts prepared, and their ability to lower plasma glucose concentration is evaluated by oral administration to a mouse model of Type 2 DM. If a glucose lowering effect is detected, the relevant extract is further purified, again given by mouth to the

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diabetic rodent, and this process continued in an effort to isolate a pure, biologically active, compound, not known to have an antihyperglycemic effect. In this presentation we will describe how this approach was used to isolate cryptolepine, pure compound from *Cryptolepis sanguinolenta,* a widely used medicinal plant in West Africa.[8,9]

Identification of *Cryptolepis sanguinolenta* as a Plant Containing an Antihyperglycaemic Compound

Cryptolepis sanguinolenta (Lindl.) Schltr. belongs to the Asclepiadaceae family that grows in several West African countries. Its common names include nimbina (Ashanti language), delboi (Fulani language), gangamau (Hausa language), nombon (Dioule language), ouidoukoi (Bambara language), and kpokpo-yangolei (Mende language), Ghana quinine, and yellow dye root. *Cryptolepis sanguinolenta* is a climbing shrub that grows commonly in disturbed open areas. The plant material used in these studies was harvested in June, September, November, and March, and herbarium specimens of the collections are deposited at the Missouri Botanical Garden and Shaman Pharmaceuticals. *Cryptolepis sanguinolenta* is used as a decoction by healers in many West African countries to treat a variety of conditions that could be associated with diabetes, such as high blood pressure and vaginal *Candida albicans* infections and is also used as a 'tonic'.[8,9] In this latter context it is often taken for years, without any evidence of side-effects or toxicity. Once a decision had been made that this was a plant worthy of further evaluation, available literature relevant to the ethnomedicine, chemistry, and biology of this plant species was evaluated by a team which included an ethnobotanist, physician, biologist, and chemist. No evidence was found describing the use of this plant or any compound isolated from this species for the treatment of diabetes. Given the promising ethnomedical data, and its apparent safety, it was selected for further evaluation.

Isolation of Cryptolepine as the Putative Glucose-lowering Compound Contained in *Cryptolepis sanguinolenta*

The *in vivo* evaluation necessary to isolate cryptolepine, the biologically active ingredient from *Cryptolepis sanguinolenta*, was performed by assessing the plasma glucose response to the oral administration of either extracts prepared from *Cryptolepis sanguinolenta* or cryptolepine itself. Male C57BL/Ks-*db/db* mice (*db/db*) or C57BL/Ks mice (normal mice), 8–9 weeks old, were dosed orally by gavage with either extract or pure compound once a day, and blood removed from the tail vein before and 3 h after administration. Plasma glucose concentrations were determined with the Glucose Diagnostic Kit from Sigma Chemical Co. (Sigma No.: 315, St Louis, MO), and plasma insulin levels determined with the Rat Insulin RIA Kit from Linco Research Inc. (Cat. no. RI-13K, St Charles, MO). For both assays, the coefficients of variation between and within assays are less than 5 %.

Figure 1 shows the effect of a 1 g kg^{-1} dose of an organic extract of *Cryptolepis sanguinolenta* on plasma glucose concentration in non-fasted *db/db* mice 3 h after dosing on days 1, 2, and 3 (time 0 represents the values before any treatment, $n = 8$). These results show that plasma glucose concentrations were significantly lower in mice receiving the plant extract on each day studied. Food intake was lower on average in mice receiving the plant extract as compared to the control (5.0 g vs 6.4 g per mouse per day), but this difference was not statistically significant due to animal variability. Body weight gain during the study was similar between mice treated with the *Cryptolepis sanguinolenta* extract or vehicle alone (0.5 vs 0.7 g per mouse).

Following this initial evidence of glucose-lowering activity, ground roots of *Cryptolepis sanguinolenta* were extracted successively with dichloromethane and aqueous sodium carbonate. The plant marc (the pulpy residue left after the juice has been pressed from the plant) was then extracted with 90 % ethanol. The ethanol extract, after concentrations, was purified using an ion exchange resin (Dowex 50X8-400). The fraction obtained from elution with 10 % ammonia in methanol was concentrated and chromatographed (HPLC) to yield an alkaloid. The alkaloid was identified as cryptolepine (5-methylidolo [3,2-b] quinoline), using one-bond and long-range, heteronuclear correlations nuclear magnetic resonance (NMR) experiments, and the assignments were in agreement with those reported in the literature.[10,11] Subsequent chemical synthesis unambiguously confirmed the structure as cryptolepine, as illustrated in Figure 2. Structurally it is quite different from other known antihyperglycaemic compounds. It should be emphasized that oral antihyperglycaemic activity in *db/db* mice was used at every step to guide the fractionation procedure from crude extract to pure compound.

Biological Activity of Cryptolepine

In Vivo Activity

Figure 3 shows changes in plasma glucose in *db/db* mice over an 8-day period in response to various daily doses of cryptolepine, the pure compound, as compared to ciglitazone. These results demonstrate a dose-dependent decline in glucose concentrations following the administration of cryptolepine. Comparison (two-way ANOVA) of cryptolepine with vehicle indicated that significant decreases in blood glucose were seen with a daily dose of both 10 mg ($p < 0.05$, $n = 8$) and 30 mg ($p < 0.01$, $n = 8$) of cryptolepine. These data also show

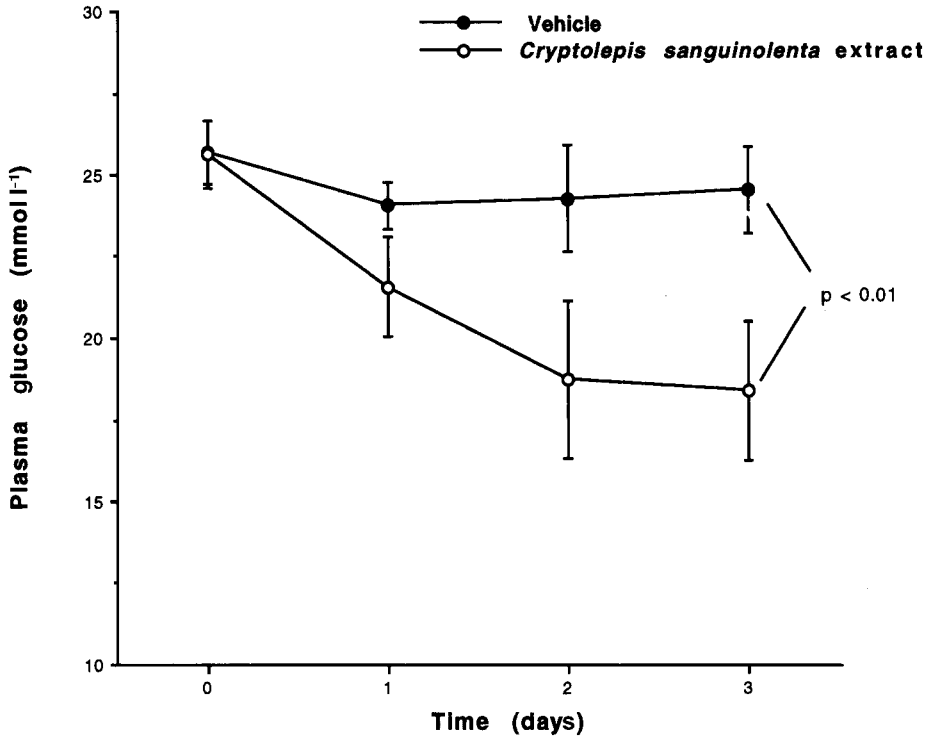

Figure 1. Effect of an oral extract of *Cryptolepis sanguinolenta* on plasma glucose concentration of *db/db* mice. There were 6 mice in each group, and two-way analysis of variance was used for statistical analysis

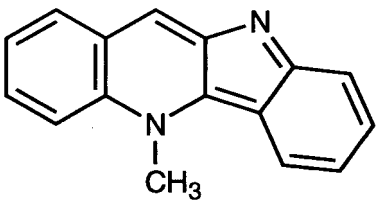

Figure 2. Chemical structure of cryptolepine

that 30 mg kg^{-1} day^{-1} of cryptolepine was approximately as effective as an equal weight dosage of ciglitazone.

Changes in plasma insulin concentration following cryptolepine and ciglitazone after 8 days of treatment are shown in Figure 4. Plasma insulin concentrations were lower than in vehicle-treated mice, in all four treatment groups, with a fall in insulin concentration of mice receiving 30 mg kg^{-1} of cryptolepine to approximately 25 % of the control value.

Table 1 contains data on daily food consumption and body weights of the mice in response to the treatment shown in Figure 3. These results indicate that although the highest dose of cryptolepine was associated with an initial decline in food consumption and body weight, this trend was reversed from days 5–8. Consequently, average food consumption was similar during the last 3 days of the study in all groups.

In order to separate further the anorexigenic from the antihyperglycaemic effect of cryptolepine, *db/db* mice were divided into a control group given free access to food, and two pair-fed groups treated with either vehicle or cryptolepine (30 mg kg^{-1}, all groups $n = 8$). Plasma glucose concentrations before and 3 days later are shown in Figure 5. It is apparent that glucose concentrations were lower in both pair-fed groups, but mice receiving cryptolepine had significantly lower plasma glucose concentrations than did the vehicle-treated group (19.9 ± 1.3 vs 24.4 ± 2.1 mmol l^{-1}, $p < 0.05$).

The fall in plasma glucose concentration following an injection of insulin intraperitoneally (0.5 U kg^{-1}) to *db/db* mice following 3 days of treatment with either vehicle ($n = 8$) or cryptolepine (10 mg kg^{-1}, $n = 8$) is seen in Figure 6. The dose of cryptolepine was selected because it had no significant effect on either glucose concentration (Figure 3) or food intake and body weight (Table 1). Although baseline plasma glucose concentrations were similar in the vehicle and cryptolepine-treated groups, the intraperitoneal administration of insulin lowered glucose concentrations to a greater degree ($p < 0.05$) in *db/db* mice treated with cryptolepine.

In Vitro Activity

Murine 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10 % (v/v) supplemented calf serum, antibiotics, and 25 mM glucose. Cells were seeded in 24-well cluster plates, grown to confluence (typically 5 days) and induced to differentiate 2 days post-confluence. Following differentiation, adipocytes were maintained in DMEM containing 10 % fetal bovine serum, provided with fresh medium every 2–3 days, and used 7–10 days post-differentiation. On the day of the experiment, adipocytes

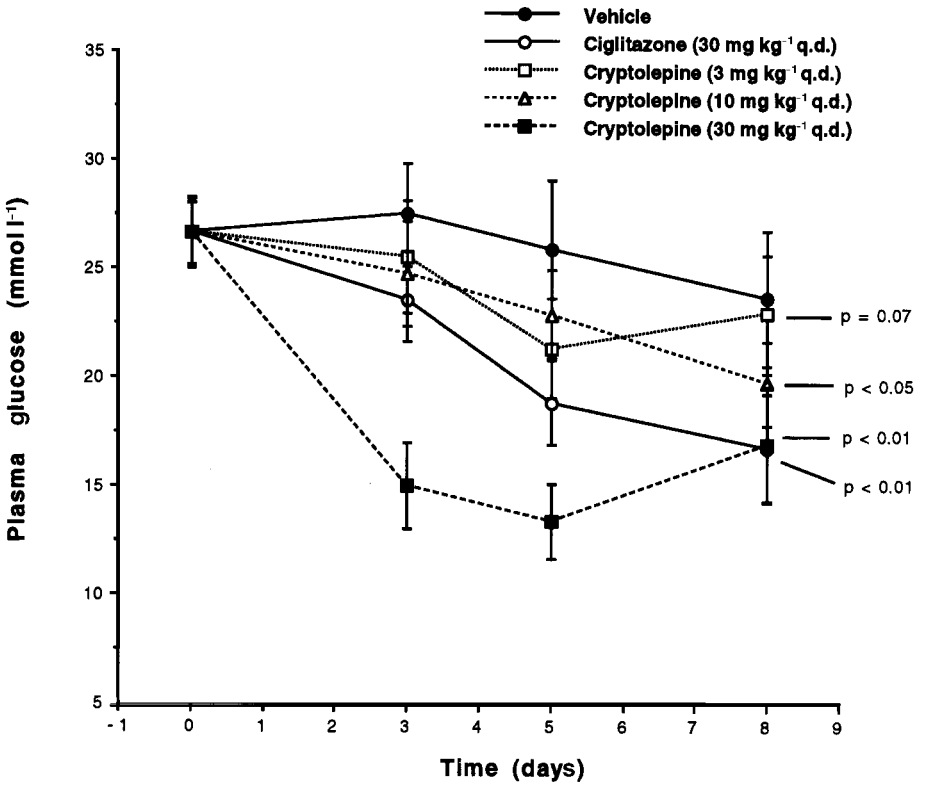

Figure 3. Comparison of the effects of cryptolepine and ciglitazone on plasma glucose concentrations of *db/db* mice. There were 8 mice in each group, and one-way analysis of variance for repeated measures was used for statistical analysis

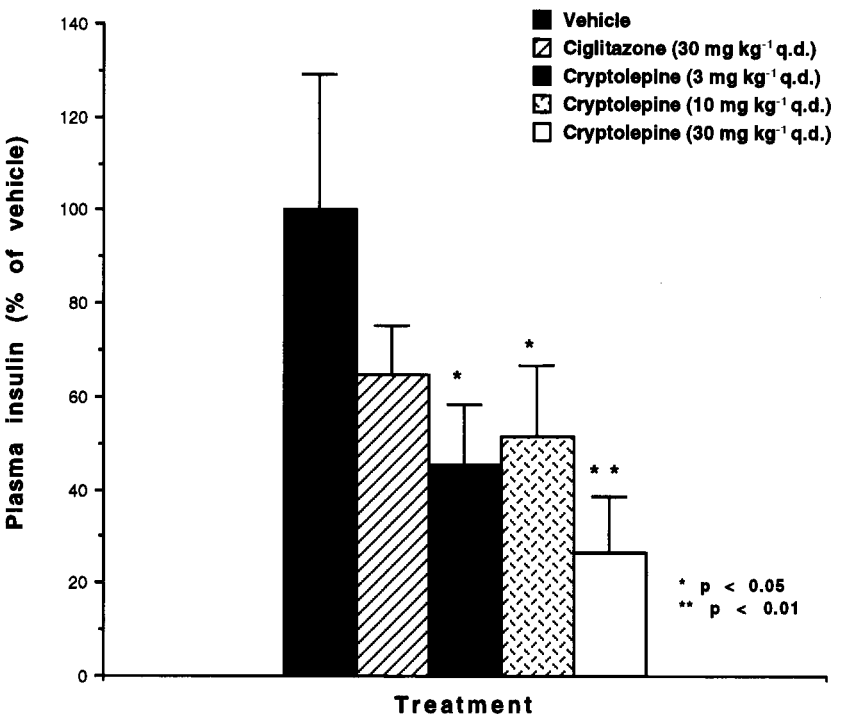

Figure 4. Plasma insulin concentrations after 8 days administration of cryptolepine or ciglitazone. There were 8 mice in each group, and one-way analysis of variance was used for statistical analysis

Table 1. Effects of cryptolepine on body weight and food intake in *db/db* mice

Measurement	Days of treatment	Vehicle	Ciglitazone 30 mg kg^{-1}	Cryptolepine 3 mg kg^{-1}	Cryptolepine 10 mg kg^{-1}	Cryptolepine 30 mg kg^{-1}
Body weight	1	40.1 ± 1.4	41.7 ± 0.7	40.8 ± 0.6	40.7 ± 1.9	39.0 ± 0.8
(g per mouse)	3	40.3 ± 1.2	42.0 ± 0.8	42.5 ± 0.8	42.1 ± 2.2	38.4 ± 0.9
	5	40.1 ± 1.1	43.2 ± 0.7	42.5 ± 0.7	42.1 ± 2.2	37.7 ± 1.1
	8	40.0 ± 1.2	43.4 ± 1.0	42.5 ± 0.8	41.8 ± 2.4	38.0 ± 1.3
Food intake	1–3	5.9	5.8	6.1	5.7	2.8
(g per mouse per day)	3–5	5.5	5.7	6.0	5.6	3.4
	5–8	4.1	4.3	4.4	4.2	4.2

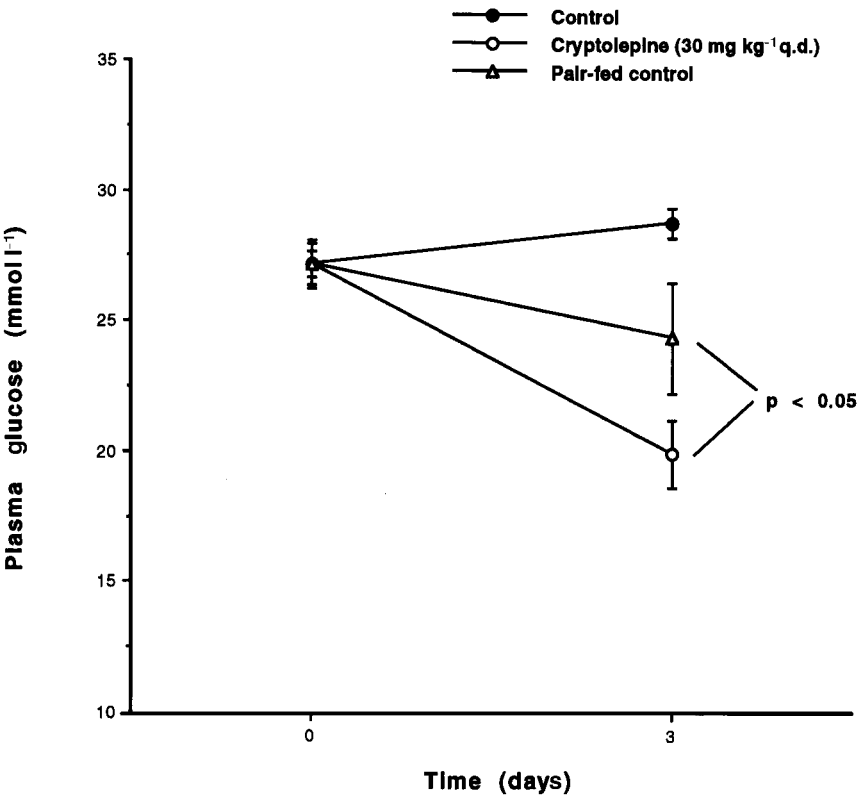

Figure 5. Plasma glucose concentrations in pair-fed *db/db* mice treated with either cryptolepine or vehicle. There were 8 mice in each group, and the difference between the pair-fed control and cryptolepine group was analysed by two-way analysis of variable

were washed with phosphate-buffered saline and switched to serum-free DMEM medium, incubated (in triplicate) for 18 h with various concentrations of cryptolepine. The culture medium was aspirated the following morning and the monolayers washed with Krebs-Ringer Hepes buffer.

To assess the effects of the compounds on basal glucose transport, 2-deoxy-d-glucose uptake was measured in the absence of insulin stimulation. To determine if 18 h exposure to compounds potentiated the stimulatory effect of insulin, adipocytes were further treated with 0.5 nM insulin (a sub-maximal concentration) for 30 min at 37 °C. Glucose transport assays were initiated by the addition of 2-deoxy-d-[^{3}H]-glucose (0.5 mCi ml^{-1}; 100 mM final concentrations) to each well followed by incubation for 10 min at 22 °C. Assays were terminated by aspirating the media and rapidly washing the monolayer twice with ice-cold phosphate-buffered saline solution. Cell monolayers were solubilized in 0.1 N NaOH, transferred to scintillation vials, and radioactivity determined by liquid scintillation counting. All data were corrected for non-specific hexose uptake determined in parallel samples treated for 5 min with 200 mM cytochalasin B.

The ability of cryptolepine to enhance glucose uptake by 3T3-L1 cIls is shown in Figure 7. These results demonstrate that cryptolepine stimulated glucose uptake in both the absence and the presence of insulin (0.5 nM), and that the effect of cryptolepine on glucose transport appears to be independent of the presence of insulin.

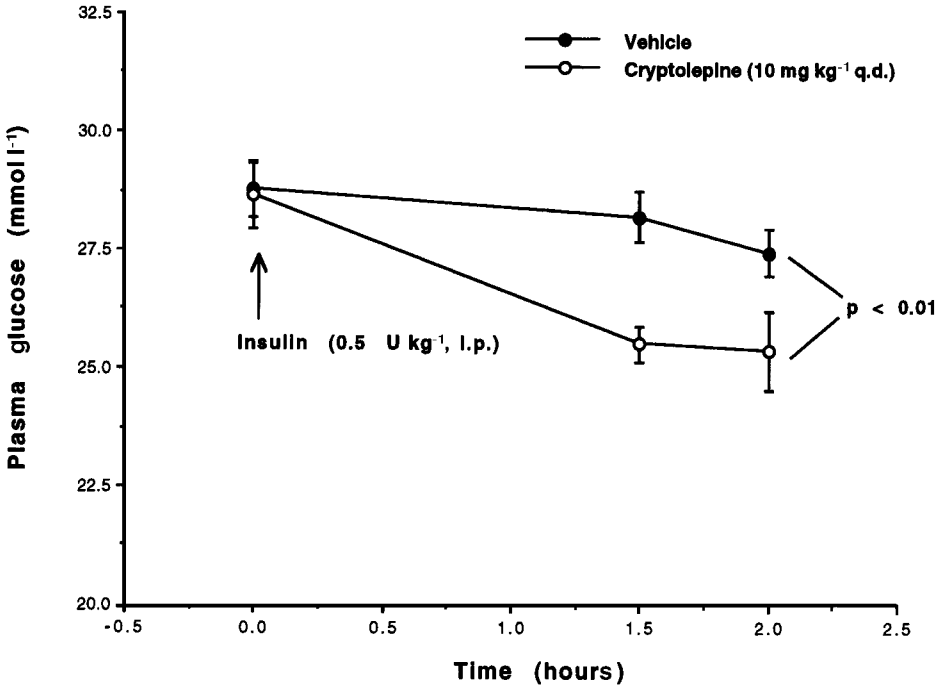

Figure 6. Comparison of the fall in plasma glucose concentration following insulin administration to *db/db* mice treated with either vehicle or cryptolepine for 3 days. There were 8 mice in each group, and two-way analysis of variance was used for statistical analysis

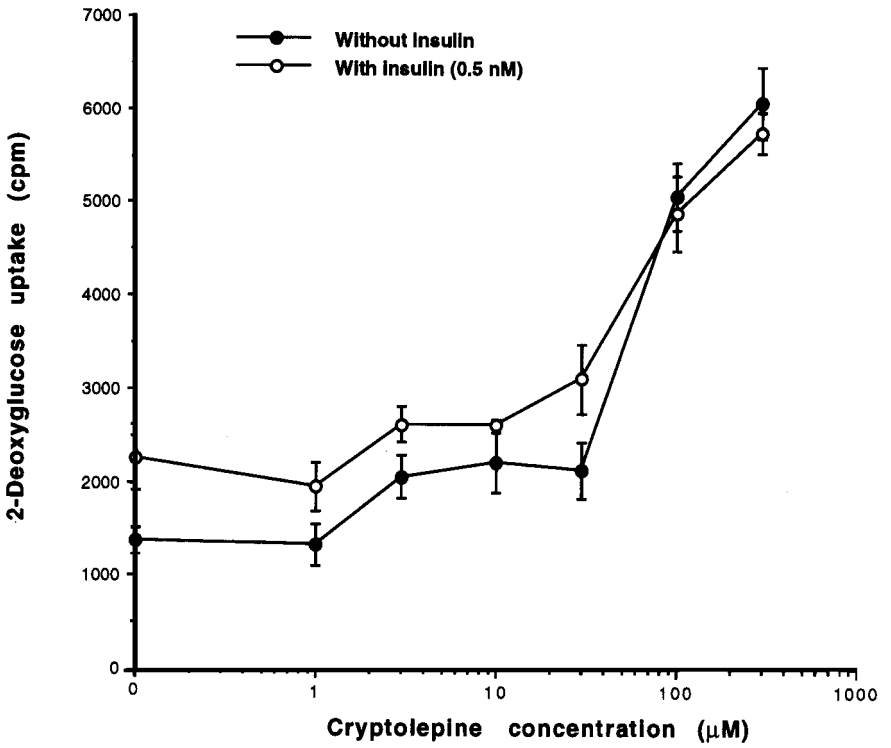

Figure 7. Effect of cryptolepine on glucose uptake by 3T3-L1 cells in culture

Studies in Patients with Type 2 DM

The decision to evaluate *Cryptolepis sanguinolenta* was based upon its widespread use in the treatment of signs and symptoms that could have been a consequence of the diabetic state. Following isolation of cryptolepine, members of the research team from Ghana indicated that it would be helpful if a small study could be initiated evaluating the metabolic effect of the plant extract as conventionally used in traditional medicine. For this purpose, 200 g of dried ground-up root was boiled in 10 l of water for 10 min and then allowed to stand at room temperature overnight. The plant marc was then removed by filtering. The final extract volume was

9.9 l. Eight newly diagnosed women with Type 2 DM volunteered for these studies, with a mean (± SD) age of 61 ± 10 years and body weight of 67.3 ± 16.8 kg. Fasting blood glucose was determined every morning using a glucometer (Lifescan, CA).

Fasting blood glucose concentration was measured for four consecutive mornings before starting treatment. During this period all patients had an overnight fasting glucose concentration > 11.1 mmol l^{-1} and the mean was 16.6 ± 1.2 mmol l^{-1}. Starting on the morning of the fourth day, patients were given 20 ml of the extract four times daily for an additional 6 days. Body weight and nocturia were recorded daily. Patients were asked to maintain their regular diet during the experimental period. All patients enrolled provided consent to participate in the study.

The results of this study are shown in Figure 8. These data indicate that plasma glucose concentrations, which were stable during a 4-day baseline period, decreased immediately after initiation of treatment with *Cryptolepis sanguinolenta* extract. By the end of the treatment period the mean fasting glucose concentration was ~ 4 mmol l^{-1} lower ($p < 0.001$). Nocturia was also reported to decline. Body weight did not change, and no adverse symptoms were reported. Chemical analysis of the extract showed that there was 92 mg l^{-1} of cryptolepine in the *Cryptolepis sanguinolenta* extract used in this study. On the basis of the daily consumption of 80 ml of extract, each of these patients consumed 7.3 mg of cryptolepine per day, equivalent to 0.11 mg kg^{-1} day^{-1} based on their mean body weight.

Discussion

We have described the potential usefulness of a drug discovery process based on integrating ethnobotany, natural product chemistry, and physiology. We have shown how it is possible to start with a plant used by indigenous healers to treat manifestations of Type 2 DM, and, by *in vivo* guided chemical fractionation, isolate a pure compound that lowers plasma glucose concentration when given by mouth to a mouse model of the disease. In addition to the belief that indigenous healers have true knowledge as to the medical usefulness of the plants they use, several other conditions must be met if this approach to drug discovery is to be effective. In the first place, the disease in question must have been present long enough for indigenous healers to have had experience with it, and the signs and symptoms of the disease apparent enough to have permitted the healer the opportunity to make a clinical diagnosis and evaluate the success of the plant remedy. We believe that both of these criteria are met if Type 2 DM is the target disease. For example, physicians trained in Ayurvedic medicine knew more than 2000 years ago that there were two types of diabetes.

Once a plant has been identified as possibly containing an interesting compound, three additional criteria must

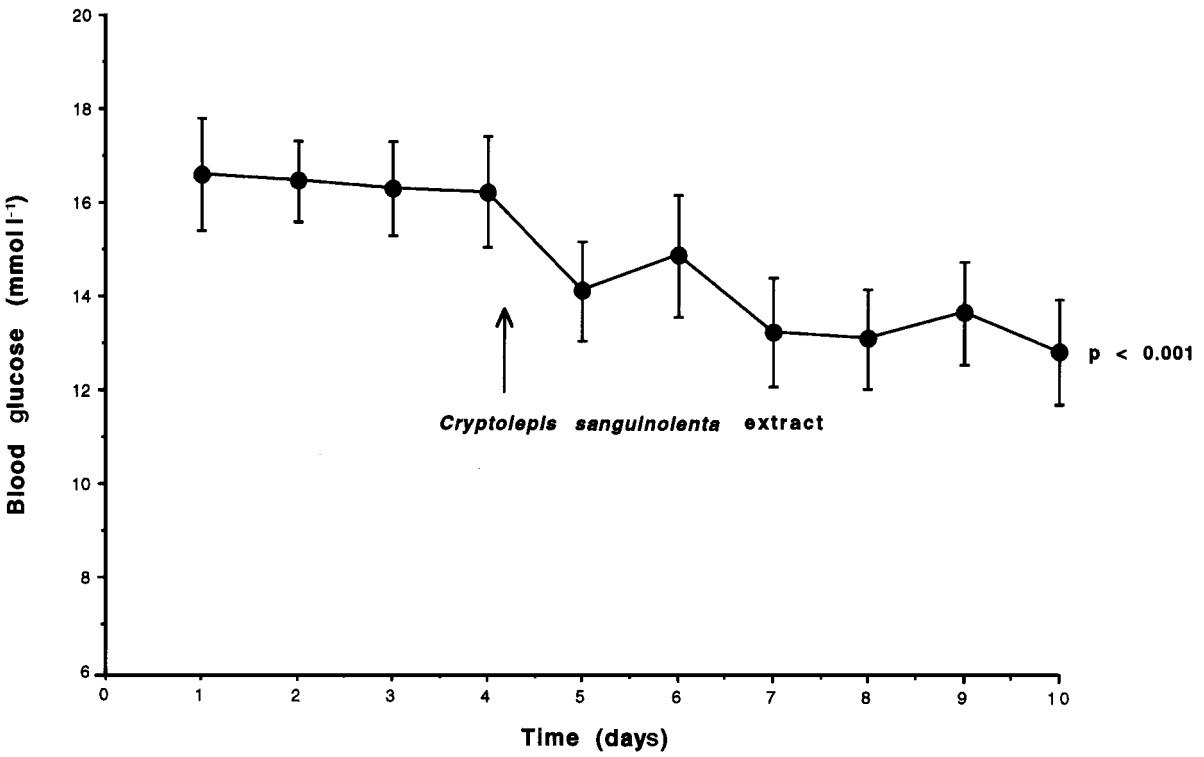

Figure 8. Fasting blood glucose concentrations in patients with Type 2 DM before and after treatment with an extract of *Cryptolepis sanguinolenta*. There were 8 patients in each group, and Student's paired *t*-test was used to compare glucose concentrations on day 10 to the mean of days 1–4

be met. Initiation of a successful fractionation programme, moving sequentially from the crude plant extract to pure compound, requires the use of a small animal model in order to conserve available material at each step of the fractionation process. It is also necessary to have an *in vivo* model with a clear-cut end-point. In the case of Type 2 DM, the *db/db* mouse satisfies both of these criteria. Modern techniques of natural product chemistry contribute the final ingredient needed to make this integrated approach successful in finding new compounds of possible clinical utility in the treatment of Type 2 DM.

Although this description of the isolation of cryptolepine from *Cryptolepis sanguinolenta* was primarily meant to serve as an example of the ethnobotanical approach to drug discovery, we have described a new compound with potential as a therapeutic agent in the treatment of Type 2 DM. Cryptolepine is an indoloquinoline alkaloid, and we are unaware of any evidence that compounds of this class affect glucose homeostasis. Cryptolepine significantly lowers plasma glucose in a mouse model of diabetes, and in this model is approximately as effective as ciglitazone. The plasma glucose-lowering effect of cryptolepine was associated with a decrease in plasma insulin concentration, and the fall in plasma glucose concentration following administration of exogenous insulin was enhanced by a dose of cryptolepine which had a marginal effect when given by itself. These results suggest that the antidiabetic effect of cryptolepine is due to an enhancement of glucose disposal, not an increase in insulin secretion; a possibility consistent with the effect of cryptolepine on glucose uptake by 3T3-L1 cells. Based upon the results in Figure 7, it appears that cryptolepine is acting directly at the cellular level to increase glucose transport, not simply to accentuate insulin-stimulated glucose uptake. This putative mechanism is not shared by any currently available anti-diabetic drug, and lends further credence to the view that cryptolepine may offer a novel approach to the treatment of Type 2 DM.

Finally, the patient studies deserve comment. In this instance, a plant extract of *Cryptolepis sanguinolenta* was administered to patients with diabetes in the same manner that is used by indigenous healers. The results shown in Figure 8 demonstrate its apparent clinical efficacy. One cannot conclude from these data that the cryptolepine in the plant extract is responsible for the fall in glucose, but the results are certainly consistent with that possibility. Perhaps of greater relevance is the quantitative evidence that plant extracts can lower glucose in diabetic patients, adding support for the view that the remedies used by native healers may well be clinically effective.

In conclusion, evidence has been presented that an ethnobotanical approach to drug discovery, based on an *in vivo* guided chemical fractionation programme, using a mouse model of Type 2 DM, can isolate and identify a pure compound present in plants that lowers glucose when given by mouth.

Acknowledgements

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